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THE INFLUENCE OF PHOTODYNAMIC SENSITIZATION ON THE ELECTRICAL AND CHEMICAL STIMULATION OF MUSCLE AND CUTANEOUS NERVE ENDINGS IN THE FROG

A DISSERTATION SUBMITTED TO THE
FACULTY OF THE DIVISION OF THE
BIOLOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGY

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THE INFLUENCE OF PHOTODYNAMIC SENSITIZATION ON THE ELECTRICAL AND CHEMICAL STIMULATION OF MUSCLE AND CUTANEOUS NERVE ENDINGS IN THE FROG¹

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FOUR FIGURES

INTRODUCTION

The photodynamic stimulation of the skeletal muscle of frogs has been studied by several investigators since the phenomenon was first described in some detail by Lippay in 1929, although Lillie and Hinrichs had observed it earlier ('23). These investigations have established several definite characteristics of this type of stimulation, namely:

1. The effective radiation falls within the absorption spectrum of the dye used (Lippay, '29).
2. Free oxygen is essential for the reaction (Lippay, '30; Spealman and Blum, '33; Lillie, Hinrichs and Kosman, '35).
3. Pure neutral Na salt solutions sensitize the stained muscles to light and this sensitization follows the Hofmeister series for anions. The Ca ion is a powerful inhibitor of the photodynamic response (Lillie et al., '35).
4. The time-intensity relationship for this type of response does not follow the Bunsen-Roscoe rule but rather seems logarithmic in nature (Lillie et al., '35).
5. Previous exposure of the stained muscle to light produces an increased irritability of the muscle to salt stimulation and to mechanical and contact influences (Lillie et al., '35).

¹ This research was aided in part by a grant from the Rockefeller Foundation.

It was to study this latter effect, increased sensitivity to stimulating agents, that the present investigation was carried out. Previously Lillie et al. ('35) had shown that if a stained muscle was exposed briefly to intense light while in Ringer's solution, it showed little or no response. But upon transferring the muscle to a pure isotonic Na salt solution (e.g., m/8 NaNO_3) it showed a markedly greater than normal response to the salt stimulation. Under such conditions the muscle was also found to be very sensitive to mechanical disturbances, such as bubbling an inert gas, nitrogen, through the solution. In a like manner there was an enhancement of contact sensitivity of the kind described by Loeb ('01). The present experiments were devised to determine whether this increased irritability of the muscle was specific to the salt stimulation or whether there was a generally increased irritability to all forms of stimulation. Electrical stimulation and further types of chemical stimulation were used.

MATERIALS AND METHODS

The experiments were performed upon the frog's sartorius. The muscles were stained in 0.1% eosin in Ringer's solution for a uniform period, 30 minutes. The source of light was a General Electric projection lantern burning a 250-watt tungsten filament lamp, giving an intensity of approximately 2000 f.c. at the site of the muscle. The light was passed through a water filter and focused on the muscle by a condensing lens. A camera shutter was placed between the lens and muscle to regulate the time of exposure.

In the electrical experiments the stimulation was carried out in a fluid bath, essentially the block type of fluid electrode as described by Rushton ('30) and modified by Chao ('35). A rectangular bakelite chamber of $12 \times 4 \times 3$ cm. was divided into two equal compartments by a partition 1 cm. thick. A glass window was placed in the long side of the chamber and through that end of the partition adjoining the window a narrow rectangular slot of 3×8 mm. connected the two compartments. The muscle was mounted in such a manner that

the nerve free pelvic end was in the slot and facing the window so that both electrical and light stimulation could be localized in this region. The chamber was so fitted with drains that the solutions could be changed rapidly and completely. The muscle was fixed at one end by the pelvic bone and at the other end attached by the tibial tendon to a writing lever. The contractions were recorded kymographically. A Zn-ZnSO_4 electrode was connected to each compartment by a Ringer-agar bridge with the cathode always at the pelvic end of the muscle. Condenser discharges were employed throughout as the method of stimulation. The resistance of the stimulating circuit was high enough (about 40,000 ohms) to render negligible any slight variations in the resistance of the muscle or solution. The stimuli were sub-maximal and throughout any particular experiment the charging voltage and condenser capacity were kept constant. Thus variations in the height of contraction became a measure of changes in the sensitivity of the muscle.

The experiments dealing with chemical stimulation were carried out in the manner described by Lillie and co-workers previously ('35). Isotonic solutions of NaCitrate and KCl were the stimulating agents used.

INFLUENCE OF LIGHT UPON THE ELECTRICAL RESPONSE

Single stimuli

The muscle was mounted in the chamber in the manner described above, and the chamber filled with Ringer's solution. The muscle was first allowed to come to equilibrium during a period of 30 to 60 minutes, after which time the height of contraction with constant stimulation remained typically constant. The charging voltage was adjusted to give a height of contraction of 50 mm. as recorded on the kymograph drum. Under such conditions the muscle is fairly sensitive to slight changes in the condenser voltage, and with constant stimulation any increase in response due to experimental treatment can be assumed to indicate a heightened irritability and decrease in response a lowered irritability. In the experiments

with single stimuli, the muscle was stimulated every 20 seconds with a single condenser discharge. When a constant response had been obtained in Ringer's solution, the chamber was rapidly drained and refilled with the Ringer's-eosin solution. The stimulation continued during the 30-minute staining period. The chamber was then emptied and washed thoroughly with Ringer's before being refilled with this solution. After a constant height of contraction had again been reached the muscle was exposed to light for several 1-second periods at intervals of 15 seconds. Suitable controls using unstained muscles were carried out and in all instances under similar conditions of exposure the unstained muscles showed no change in height of contraction upon exposure to light.

In table 1 the results of these experiments are shown. In fifty-three experiments in which the response to electrical

TABLE 1
Influence of light upon electrical irritability of muscle.
(Using single condenser discharges)

NUMBER OF EXPERIMENTS	PER CENT INCREASE IN HEIGHT OF CONTRACTION AFTER EXPOSURE	CONTROL HEIGHT IN MILLIMETERS
5	52% (average)	50
12	10	50
36	0	50

stimulation was constant, and the muscle normally sensitive, only seventeen showed any increase in the height of contraction after exposure to light and of this number only five were definitely positive, that is, the increase in height was relatively great and comparable to the increases obtained with salt stimulation. These results show a marked contrast with the great increase in sensitivity to salt solutions demonstrated by Lillie and co-workers ('35). In the experiments described above, continued exposure to light resulted in the typical decrease of irritability induced by prolonged exposure, as was also shown by Lillie et al. ('35). Any increase in irritability is a temporary one, the continued exposure to light resulting in irreversible destructive changes in the muscle. This statement holds true both of salt and electrical stimulation.

Repeated stimuli

Since in salt stimulation the muscle is constantly being bombarded by the agents of stimulation and since in stimulation by single discharges as above this is not the case, the type of stimulation was changed so as to correspond more closely in its general character to salt stimulation. Instead of single discharges a succession of discharges repeated at a regular rate of about 60 per minute (using a metronome) was used to stimulate. Stimulation was continued until response was greatly reduced by fatigue, i.e., for about 1 minute. The muscle was then rested for 15 minutes. The maximum height of contraction obtained under these conditions was taken as the standard of measurement. The unstained muscle was

TABLE 2

*Influence of light upon electrical irritability of muscle.¹
(Using repeated condenser discharges at 60 per minute)*

CONTROL HEIGHT IN MILLI- METERS	HEIGHT AFTER 10 MINUTES IN EOSIN (IN DARK)	HEIGHT AFTER 30 MINUTES IN EOSIN (IN DARK)	HEIGHT OF STAINED MUSCLE IN RINGER BEFORE EXPOSURE	NUMBER OF SECONDS EXPOSURE TO LIGHT	HEIGHT OF CONTRAC- TION AFTER EXPOSURE TO LIGHT
31.6	22.3	14.5	4.5	21	52

¹ Averages of twenty-one experiments.

stimulated in this manner until reproducible contraction curves were obtained. The muscle was then stained in the manner outlined above and Ringer's solution again placed in the chamber. Throughout the experiment the muscle was stimulated every 15 minutes. When fairly constant heights of contraction had been obtained after staining, the muscle was exposed to the light in the manner described above. The results of exposure of the unstained muscle to light were uniformly negative. In table 2 are shown the results of these experiments and in figure 1 the record of a typical experiment.

With this repeated type of stimulation the results were very different. Although only twenty-one experiments have been carried out so far, seventeen of these were conclusively positive, showing a significant increase in the height of contraction

after exposure to light. This increase was obtained only when the exposure took place before stimulation. Exposing the muscle to light during stimulation was ineffective. This increased height of contraction was not long maintained; as a rule, after 15 minutes the muscle had lost its hyperirritability. This rapid decline of responsiveness to repeated electrical stimuli is characteristic for electrical stimulation of photosensitized muscle, and is in contrast to the increased sensitivity shown to salt stimulation, which is maintained for a considerable time (Lillie et al., '35). This difference of behavior may be related to the much longer exposure periods required to sensitize the muscle to electrical stimulation. About twenty-one exposures of 1 second each (with intervals of 5 seconds), are necessary in the case of electrical stimulation as compared to five or six 1-second exposures for the salt stimulation. In the case of the former the destructive effects of the light may intervene at an earlier time.

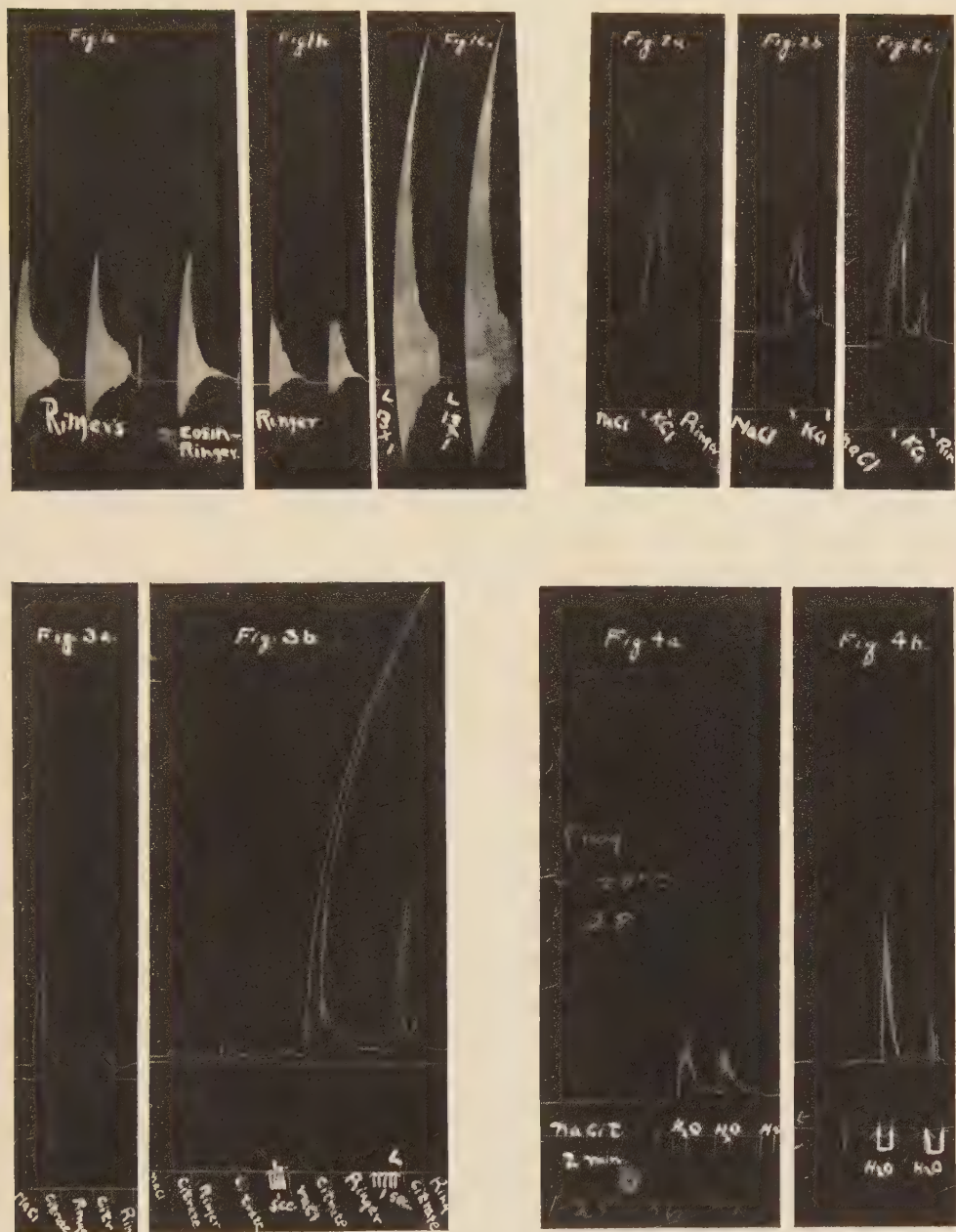
The effect of eosin alone on the muscle in the absence of light is to decrease the response to all forms of stimulation. As suggested by Lillie et al. ('35) this desensitizing effect is probably to be referred to adsorption of the dye on the membrane colloids, resulting in an increased stability of these colloids. The increased sensitivity of the stained muscle to

Fig. 1 Effectiveness of photodynamic stimulation in increasing the response of muscle to electrical stimulation. a, b, response of unstained muscle in Ringer's and during staining in Ringer's-eosin. c, response of stained muscle immediately after exposure to light. Numbers refer to number of 1-second exposures given to muscle, that is, $L\ 13 \times 1 =$ thirteen 1-second exposures at intervals of 15 seconds.

Fig. 2 Effectiveness of photodynamic stimulation in increasing response of muscle to K stimulation. a, response of unstained muscle to K stimulation. b, response of stained muscle in dark before exposure to light. c, response after exposure to light for six 1-second periods.

Fig. 3 Same as figure 2 but using NaCitrate. a, response of unstained muscle to citrate stimulation. b, response of stained muscle before exposure to light and after exposure. Each arrow denotes an exposure period of 1 second.

Fig. 4 Enhancement of 'contact' stimulation of skin sensory endings by means of previous exposure to light. a, withdrawal response of unstained limb from H_2O after 2 minutes immersion in NaCitrate. b, same response after staining and exposing to light.



electrical and salt stimulation after exposure to light is in striking contrast to the depressant effect of the dye itself. Exposure to light under the conditions already described is the only effective remover of this state of depression or inhibition; repeated washing of the stained muscle in Ringer's solution in the dark is without effect. There is a difference here to certain other types of photodynamic effects; for example, in hemolysis by eosin the effect of the dye in the dark is similar to the effect in the light, only higher concentrations and longer exposures are required (Blum, '32, '35).

POTASSIUM AND CITRATE STIMULATION

The muscle was immersed in beakers containing the various solutions and attached to a light isotonic lever for graphic recording. The experimental solutions could be readily substituted for one another without disturbing the muscle. The typical procedure was as follows: a muscle was mounted and immersed in Ringer's; the Ringer's was then replaced by a solution of 15 parts m/8 NaCl and 1 part m/8 KCl. The muscle was then returned to Ringer's for 5 minutes after which pure m/8 NaCl was substituted for it. After being sensitized in this solution for 2 minutes the muscle was again exposed to the stimulating solution. The height of contraction in the stimulating solution was the standard of measurement. The muscle was treated in this way before and after staining in order to obtain reproducible controlled stimulations. Exposure of the unstained muscle to light gave uniformly negative results, that is, the height of contraction in the stimulating solution was unaffected by light. In the citrate series the procedure was identical except that isotonic NaCitrate was used for stimulation instead of KCl. The results of both these series is shown in table 3 (averages of all experiments), the record of typical experiments in figures 2 and 3.

The results were definitely positive in these experiments, and similar to those obtained by Lillie and co-workers ('35), using pure salt solutions as the stimulating agents. The effect of the dye in the dark upon the response was again a marked

depressant one; the depression was quickly removed by a few exposures of 1 second each. The increase in response was very marked and persisted even after 30 minutes without any further exposure to light. In summary, these results were completely in accord with those obtained by Lillie using the lyotropic anion series ('35).

TABLE 3

Influence of light upon the potassium and citrate stimulation of muscle

SALT	NUMBER OF EXPERIMENTS	CONTROL HEIGHT IN MILLIMETERS AFTER		CONTRACTION HEIGHT OF STAINED MUSCLE IN DARK AFTER		SECONDS EXPOSURE	HEIGHT AFTER EXPOSURE AFTER	
		Ringer's	NaCl	Ringer's	NaCl		Ringer's	NaCl
KCl	13	9	40	0	18	7	51	110
NaCitrate	16	5	28	0	13	8	70	116

STIMULATION OF SKIN SENSORY ENDINGS

Blum and Spealman had shown in 1934 that frogs' skin could be sensitized to light by means of suitable dyes. A frog injected with eosin will upon exposure to light show signs of general excitation and with extreme exposure, paralysis and death. This effect is abolished after destruction of the brain and spinal cord. A spinal frog having one of its limbs stained externally will show the flexor reflex when the limb is exposed to light. In the present series of experiments the frog was stained both by injection into the dorsal lymph-sac and by external staining of the limb, the results being similar in both cases. The leg of the frog was then immersed in isotonic sodium citrate or aluminium chloride solutions for 3 minutes, and then dipped in a beaker containing distilled water. A strong withdrawal reflex is the result. The flexing of the limbs in the distilled water was recorded by means of a muscle lever. This is a typical contact sensitization phenomenon, as previously described by Loeb ('01). The influence of exposure to light upon the withdrawal response from the distilled water was then studied. Table 4 shows the results of these experiments. Figure 4 gives a typical record. It is evident that the

exposure to light resulted in an increased irritability of the sensory endings to this type of stimulation. In this respect the general result is similar to that obtained with the sartorius muscle; that is, the effect of eosin in the presence of strong light is to increase the sensitivity of the cutaneous endings.

TABLE 4
Influence of light upon the stimulation of skin sensory endings by NaCitrate and $AlCl_3$

SALT	NUMBER OF EXPERIMENTS	CONTROL HEIGHT IN MILLIMETERS	HEIGHT AFTER EXPOSURE OF STAINED SKIN TO LIGHT	PER CENT INCREASE	SECONDS EXPOSURE
NaCitrate	16	14	27	93	15-45
$AlCl_3$	12	33	43	30	15-45

DISCUSSION

These experiments tend to confirm the suggestions put forward by Lillie et al. ('35), and Kosman and Lillie ('35), that the primary effect of the light is upon the membrane colloids, probably the proteins, resulting in some alteration of structure or properties in the general direction of decreased stability. Under certain conditions this instability may lead to stimulation, e.g., in low Ca concentrations. However, under conditions where there is no stimulation process set in motion, the altered state of the membrane colloids may persist; the result is an enhancement of the effect produced by a subsequently acting stimulating agent. In the experiments reported here the most effective stimulating agents for the photosensitized muscle have been pure solutions of Na salts of the lyotropic series and the least effective, electrical stimulation.

SUMMARY

1. Exposure of eosin-stained muscles to light for sufficient time periods increases the response of the muscle to repeated electrical stimulation, but not (or slightly) to single stimuli.
2. The chemical irritability of eosin-stained muscle as determined by KCl and NaCitrate stimulation is increased after exposure to light.

3. The effect of the dye alone, in the dark, upon chemical and electrical irritability is a depressant one.

4. The irritability of eosin-stained skin sensory endings to 'contact' stimulation is increased after exposure to light.

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